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Upregulation by retinoic acid of transforming growth factor-β-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases

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Abstract

We investigated whether transforming growth factor-β (TGF-β) stimulates the induction of heat shock protein (HSP) 27 and HSP70 in osteoblast-like MC3T3-E1 cells and the mechanism underlying the induction. TGF-β increased the level of HSP27 but had no effect on the HSP70 level. TGF-β stimulated the accumulation of HSP27 dose-dependently, and induced an increase in the level of mRNA for HSP27. TGF-β induced the phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase. The HSP27 accumulation induced by TGF-β was significantly suppressed by PD98059, an inhibitor of the upstream kinase of p44/p42 MAP kinase, or SB203580, an inhibitor of p38 MAP kinase. PD98059 and SB203580 suppressed the TGF-β-stimulated increase in the level of mRNA for HSP27. Retinoic acid, a vitamin A (retinol) metabolite, which alone had little effect on the HSP27 level, markedly enhanced the HSP27 accumulation stimulated by TGF-β. Retinoic acid enhanced the TGF-β-induced increase of mRNA for HSP27. The amplification of TGF-β-stimulated HSP27 accumulation by retinoic acid was reduced by PD98059 or SB203580. Retinoic acid failed to affect the TGF-β-induced phosphorylation of p44/p42 MAP kinase or p38 MAP kinase. These results strongly suggest that p44/p42 MAP kinase and p38 MAP kinase take part in the pathways of the TGF-β-stimulated HSP27 induction in osteoblasts, and that retinoic acid upregulates the TGF-β-stimulated HSP27 induction at a point downstream from p44/p42 MAP kinase and p38 MAP kinase.

Keywords: Transforming growth factor-β; Heat shock protein; Osteoblast; Mitogen-activated protein kinase; Retinoic acid

1. Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates cell growth, differentiation and extracellular matrix production [1]. Bone metabolism is maintained by two types of functional bone cells, osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively [2]. In bone tissue, TGF-β is produced by osteoblasts, and TGF-β is abundantly

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stored as a latent form in bone matrix tissue [3]. TGF-β is released by osteoclasts as an active TGF-β and the active TGF-β could act to both stimulate new bone formation and limit the extent of further bone resorption [3]. TGF-β stimulates the recruitment and proliferation of osteoblasts responsible for new bone formation [3]. In osteoblasts, it has been reported that TGF-β regulates α1(I) collagen gene expression, and causes the induction of osteoprotegerin/osteoclastogenesis inhibitory factor mRNA, and induces vascular endothelial growth factor expression [4–6]. Taken together, TGF-β is well recognized to be an important cytokine in bone metabolism.

The intracellular signaling of TGF-β is initiated following ligand binding to the TGF-β type II receptor, which phosphorylates TGF-β type I receptor resulting in the activation of type I receptor [7,8]. It is generally recognized that the activated TGF-β type I receptor phosphorylates Smad 2 and Smad 3. Phosphorylated Smad 2 and Smad 3 form a complex with Smad 4, and these complexes translocate into the nucleus where they can bind to DNA in the promoters of TGF-β target genes or interact with DNA-binding proteins to form a transcriptional activator complex [9–12]. In addition to this signaling pathway, other signaling pathways such as the mitogen-activated protein (MAP) kinase superfamily have recently been shown to mediate TGF-β signaling. Three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and c-*Jun* N-terminal kinase (JNK) are known as central elements used by mammalian cells to transduce the diverse messages [13]. TGF-β-activated kinase (TAK1), a member of the MAP kinase kinase kinase family, has been identified as upstream kinase of MAP kinase [14]. The kinase activity of TAK1 is stimulated by TGF-β in osteoblast-like MC3T3-E1 cells [14]. TGF-β reportedly activates the p44/p42 MAP kinase pathway in primary cultured arterial endothelial cells [15]. Additionally, it has been shown that TGF-β activates the p38 MAP kinase pathway and p38 MAP kinase is involved in the TGF-β-induced transcriptional activation by regulating the Smad-mediated pathway in C2C12 pluripotent mesenchymal precursor cells [16]. However, the exact roles of the MAP kinase superfamily in the TGF-β signaling system in osteoblasts are still poorly understood.

Cells produce heat shock proteins (HSPs), when exposed to biological stress such as heat [17]. HSPs are classified into high molecular weight HSPs and low molecular weight HSPs according to apparent molecular sizes. High molecular weight HSPs such as HSP110, HSP90 and HSP70 are well recognized as acting as molecular chaperones in protein folding, oligomerization and translocation [18]. Though the functions of low molecular weight HSPs are less known than those of high molecular weight HSPs, it is recognized that they also may act as molecular chaperones like high molecular weight HSPs [17]. In osteoblasts, the expression of HSP27 is induced by heat, and the heat-induced HSP27 expression is reportedly facilitated by estrogen [19,20]. In addition, the downregulation of proliferation has been shown to be accompanied by a transient increase of the expression of HSP27 mRNA [19,20]. However, the exact mechanism behind the HSP27 induction in osteoblasts and its roles have not yet been precisely clarified. As for TGF-β effects on HSPs in osteoblasts, it has been reported that TGF-β induces HSP27 phosphorylation in osteoblast-like MC3T3-E1 cells [21], and TGF-β causes an increase in the steady state level of HSP47 mRNA in these cells [22].

Steroid hormones such as thyroid hormone, vitamin D_3, estrogen, and retinoic acid consist of the steroid hormone receptor superfamily of ligand-inducible transcription factors [23]. It is well known that the effects of steroid hormones are exerted via binding to specific intracellular receptors and subsequently activating the expression of gene network [23]. Among them, retinoic acid, a vitamin A (retinol) metabolite, has marked effects on proliferation and differentiation of a variety of cells [23]. The receptors for retinoic acid have been demonstrated to exist in osteoblast-like MC3T3-E1 cells [24]. As for bone metabolism, retinoic acid is recognized to act as a bone resorptive agent [25]. Accumulating evidence indicates that steroid hormones modulate intracellular signaling by other agonists [24–26]. Recently, it has been shown that the ligand-dependent interaction between Smad 3 and retinoid X receptor/vitamin D receptor heterocomplex is observed and the cooperative actions of TGF-β and vitamin D signaling pathways are mediated by Smad 3 [28]. Thus, we thought it possible that retinoic acid might modulate TGF-β signaling in osteoblast-like MC3T3-E1 cells.

In the present study, we investigated the effects of TGF-β on the induction of HSP27 and HSP70 in MC3T3-E1 cells and the potential cross-talk between TGF-β signaling and retinoic acid signaling. We here report that both p44/p42 MAP kinase and p38 MAP kinase take part in TGF-β-stimulated HSP27 induction in these cells and that retinoic acid amplifies the HSP27 induction by TGF-β.

2. Materials and methods

2.1. Materials

TGF-β and epidermal growth factor (EGF) were obtained from R&D Systems (Minneapolis, MN, USA). Anisomycin was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Inducible HSP70 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). PD98059, SB203580 and PD169316 were obtained from Calbiochem-Novabiochem. All *trans* retinoic acid was purchased from Sigma (St. Louis, MO, USA). Phospho-specific p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), and p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific JNK antibodies (rabbit polyclonal IgG, affinity purified) and phospho-specific Smad 2 antibodies were purchased from New England BioLabs (Beverly, MA, USA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PD98059, SB203580 and PD169316 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1% (v/v), which did not affect Western blot analysis, immunoassay of HSP27 or Northern blot analysis of HSP27.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [29] were maintained as previously described [30]. In brief, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% (v/v) fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35 mm diameter dishes or 90 mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with PD98059, SB203580 or PD169316 for 60 min. The pretreatment of retinoic acid was performed for 8 h.

2.3. Western blot analysis of HSP27, HSP70, p44/p42 MAP kinase and p38 MAP kinase

The cultured cells were stimulated by TGF-β in α-MEM for the indicated periods. For heat treatment, dishes containing cells were floated in a water bath at 43°C for 30 min and then returned to the incubator for 12 h. When indicated, the cultured cells were stimulated by EGF (30 ng/ml) for 10 min or anisomycin (100 nM) for 20 min, respectively. The cells were washed twice with phosphate-buffered saline (PBS) and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at $125\,000 \times g$ for 10 min at 4°C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [31] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [32] using HSP27 antibodies, HSP70 antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-JNK antibodies or phospho-specific Smad 2 antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

2.4. Immunoassay of HSP27

The concentration of HSP27 in soluble extracts of cells was determined by sandwich-type enzyme immunoassays, as described previously [33]. The cul-

tured cells were stimulated by TGF-β in α-MEM for the indicated periods. The cells were then washed twice with 1 ml of PBS, and frozen at −80°C for a few days before analysis. The frozen cells on each dish were collected and suspended in 0.3 ml of PBS, and each suspension was sonicated and centrifuged at 125 000×*g* for 20 min at 4°C. The supernatant was used for the specific immunoassay of HSP27. In brief, we used an enzyme immunoassay system that employs polystyrene balls (3.2 mm in diameter, Immuno Chemicals, Okayama, Japan) carrying immobilized $F(ab')_2$ fragments of antibody and the same Fab′ fragments labeled with β-D-galactosidase from *Escherichia coli*. A polystyrene ball carrying antibodies was incubated either with the purified standard for HSP27 or with an aliquot of the samples. This incubation was carried out at 30°C for 5 h in a final volume of 0.5 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl, 0.5% (w/v) hydrolyzed gelatin, 0.1% (w/v) bovine serum albumin (BSA), 1 mM $MgCl_2$ and 0.1% (w/v) NaN_3. After washing, each ball was incubated at 4°C overnight with 1.5 milliunits of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA and 0.1% NaN_3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl β-D-galactoside.

2.5. Isolation of RNA and Northern blot analysis of HSP27

The cultured cells were stimulated by TGF-β in α-MEM for the indicated periods. Total RNA was isolated using a QuickPrep Total RNA Extraction kit (Pharmacia Biotech, Tokyo, Japan). Then, 20 μg of total RNA were subjected to electrophoresis on a 0.9% agarose–2.2 M formaldehyde gel, and blotted onto a nitrocellulose membrane. For Northern blot analysis, the membrane was allowed to hybridize with a cDNA probe that had been labeled using a Multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously [32]. A *Bam*HI-*Hin*dIII fragment of cDNA for mouse HSP27 [20] was kindly provided by Dr. L.F. Cooper of the University of North Carolina.

2.6. Sucrose density gradient centrifugation

The cultured cells were exposed to heat (43°C, 30 min). After 12 h, the cells were stimulated by TGF-β. Extract of the cells was layered over a 3.5 ml linear gradient of sucrose (10–40%) in 50 mM Tris–HCl, pH 7.0, that contained 5 mM EDTA and was centrifuged at 130 000×*g* for 16 h at 4°C in a swinging bucket rotor (RPS56T, Hitachi). After centrifugation, each sample was fractionated into 15 test tubes, each of which contained 0.25 ml of 0.1% BSA.

2.7. Other methods

Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA), with BSA as the standard protein. Rat HSP27, which was used as the standard for the immunoassay, was purified from skeletal muscle [33]. The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad).

2.8. Statistical analysis

Each experiment was repeated three times with similar results. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $P < 0.05$ was consid-

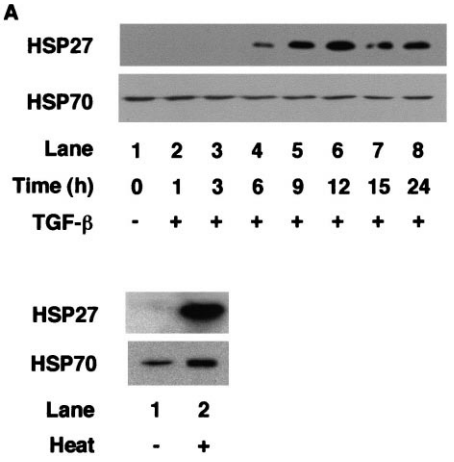


Fig. 1. Effects of TGF-β on HSP27 and HSP70 induction in MC3T3-E1 cells. The cultured cells were stimulated by 3 ng/ml TGF-β for the indicated periods. The extracts of cells were subjected to SDS–PAGE against HSP27 antibodies or HSP70 antibodies. Lower blots show the positive controls for the induction of HSP27 and HSP70 by heat (43°C, 30 min).

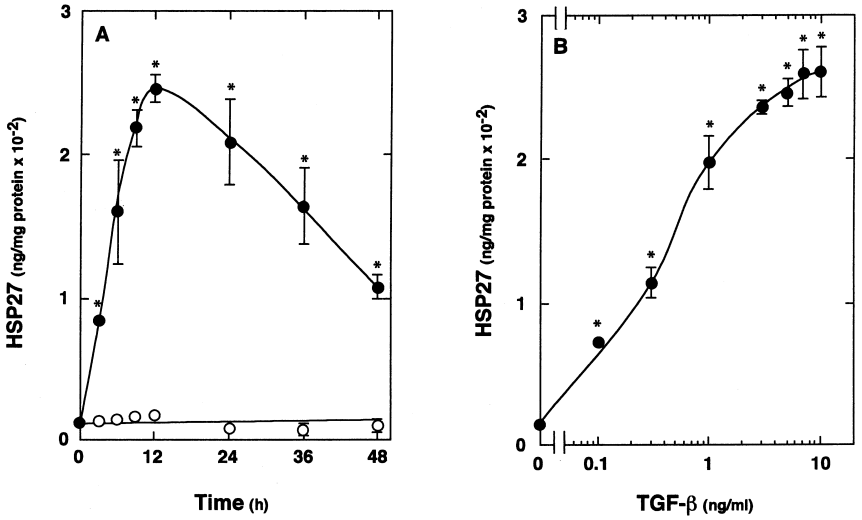


Fig. 2. Effect of TGF-β on HSP27 accumulation in MC3T3-E1 cells. (A) The cultured cells were stimulated by 3 ng/ml TGF-β (●) or vehicle (○) for the indicated periods. (B) The cultured cells were stimulated by various doses of TGF-β for 12 h. Each value represents the mean ± S.D. of three different sample sets. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the control value.

ered significant. All data are presented as the mean ± S.D. of three different sample sets.

3. Results

3.1. Effects of TGF-β on HSP27 and HSP70 induction in MC3T3-E1 cells

In unstimulated MC3T3-E1 cells, the level of HSP27 was quite low. On the other hand, HSP70 clearly exists. TGF-β (3 ng/ml) time-dependently increased the level of HSP27 and the maximum level of HSP27 was observed at 12 h after stimulation. However, TGF-β did not affect the level of HSP70 in the same sample (Fig. 1). We showed the induction of HSP27 or HSP70 by heat shock as a loading positive control. HSP27 and HSP70 were truly induced by heat in osteoblast-like MC3T3-E1 cells.

3.2. Effect of TGF-β on HSP27 accumulation in MC3T3-E1 cells

To clarify the effect of TGF-β on HSP27 induction in MC3T3-E1 cells, we determined the level of HSP27 by a specific immunoassay. TGF-β (3 ng/ml) significantly stimulated the accumulation of HSP27 time-dependently up to 48 h (Fig. 2A). The maximum effect of TGF-β on HSP27 accumulation was observed at 12 h after stimulation and the level of HSP27 decreased thereafter. The stimulative effect of TGF-β on HSP27 accumulation was dose-dependent in the range between 0.1 and 10 ng/ml (Fig. 2B). The maximal effect of TGF-β on HSP27 accumulation was observed at 10 ng/ml.

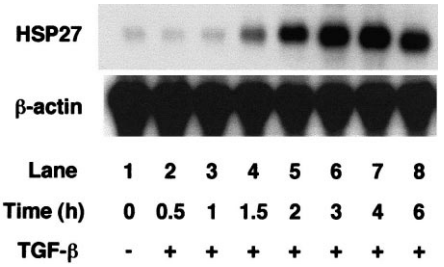


Fig. 3. Northern blot analysis of the mRNA for HSP27 in response to TGF-β in MC3T3-E1 cells. The cultured cells were stimulated by 3 ng/ml TGF-β for the indicated periods. The cells were then harvested and total RNA was isolated. Twenty micrograms of RNA from each sample were subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with the cDNA probe for HSP27 or β-actin. Lane 1, control cells. Bands of β-actin are shown for reference.

3.3. Northern blot analysis of the mRNA for HSP27 in response to TGF-β in MC3T3-E1 cells

The expression level of the mRNA for HSP27 was markedly increased by TGF-β, which had little effect on the level of β-actin mRNA (Fig. 3). The maximal effect of TGF-β on the mRNA level for HSP27 was observed at 4 h after stimulation.

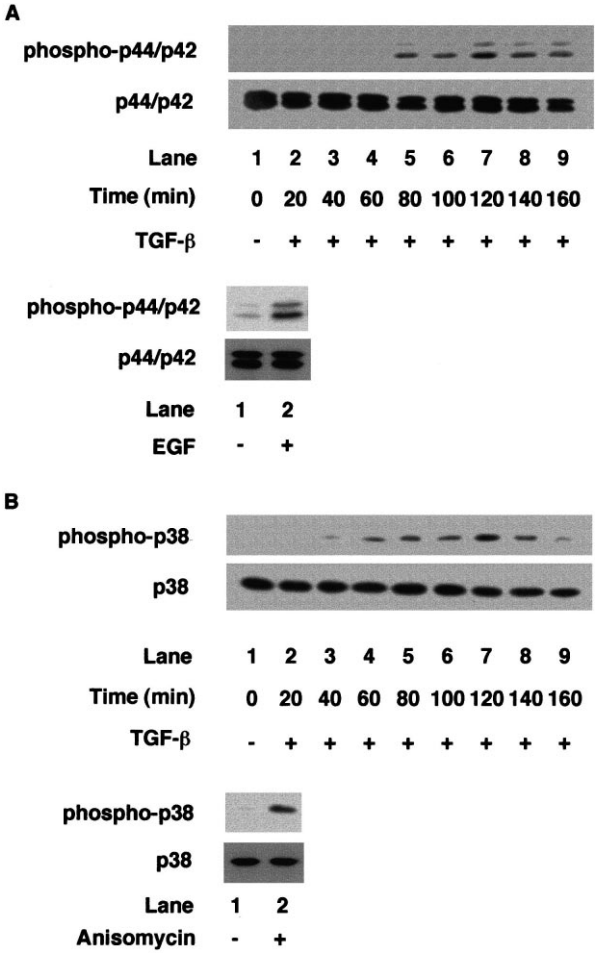


Fig. 4. Effects of TGF-β on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 3 ng/ml TGF-β for the indicated periods. The extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. Each lower blot shows the positive control for the EGF-induced phosphorylation of p44/p42 MAP kinase or the anisomycin-induced phosphorylation of p38 MAP kinase.

3.4. Effects of TGF-β on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells

To investigate whether or not TGF-β activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells, we next examined the effect of TGF-β on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase. TGF-β markedly induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in a time-dependent manner (Fig. 4). We showed the phosphorylation of p44/p42 MAP kinase by EGF and the phosphorylation of p38 MAP kinase by anisomycin, a direct activator of p38 MAP kinase, as loading positive controls.

3.5. Effects of PD98059, SB203580 and PD169316 on TGF-β-induced HSP27 accumulation in MC3T3-E1 cells

In order to investigate whether or not p44/p42 MAP kinase is involved in the TGF-β-stimulated HSP27 induction in MC3T3-E1 cells, we examined the effect of PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase [34], on the HSP27 accumulation stimulated by TGF-β. PD98059, which alone had little effect on the basal level of HSP27, significantly reduced HSP27 accumulation by TGF-β (Fig. 5, left). The inhibitory effect of PD98059 on the TGF-β-induced HSP27 accumulation was dose-dependent in the range between 0.1 and 30 μM. The maximal effect of PD98059 was observed at 30 μM, a dose that caused about 50% reduction in the effect of TGF-β (Fig. 5, left). We next investigated the involvement of p38 MAP kinase in the TGF-β-stimulated HSP27 induction in MC3T3-E1 cells. SB203580, an inhibitor of p38 MAP kinase [35], which by itself did not affect the basal level of HSP27, markedly suppressed the HSP27 accumulation induced by TGF-β in a dose-dependent manner in the range between 0.1 and 30 μM. The maximal effect of SB203580 was observed at 30 μM, a dose that caused about 80% reduction in the effect of TGF-β (Fig. 5B). Moreover, we examined the effect of PD169316, another inhibitor of p38 MAP kinase [36], on the accumulation of HSP27 induced by TGF-β. PD169316 (10 μM), which alone had little effect on the level of HSP27, markedly re-

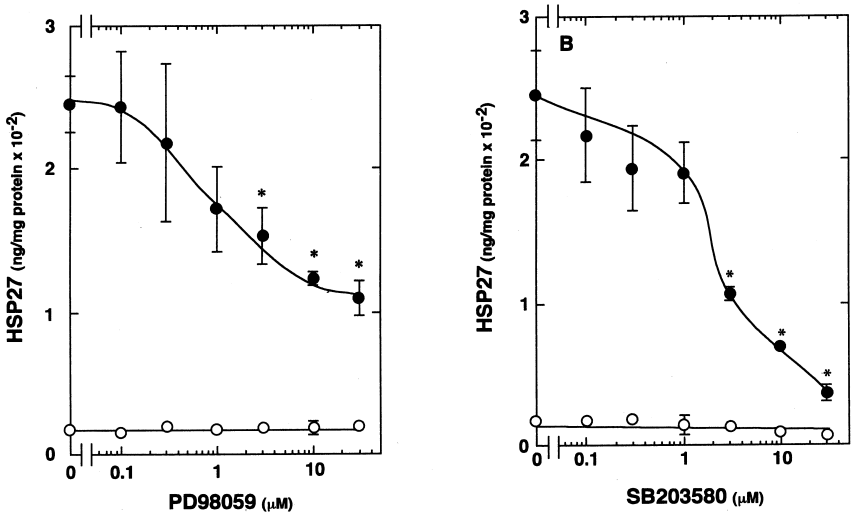


Fig. 5. Effects of PD98059 and SB203580 on TGF-β-induced HSP27 accumulation in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 (left) or SB203580 (B) for 60 min, and then stimulated by 3 ng/ml TGF-β (●) or vehicle (○) for 12 h. Each value represents the mean ± S.D. of three different sample sets. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of TGF-β alone.

duced the HSP27 accumulation (control, 13.7 ± 1.3 ng/mg protein; TGF-β alone, 245.7 ± 24.6 ng/mg protein; 10 μM PD169316 alone, 10.7 ± 1.5 ng/mg protein; TGF-β plus 10 μM PD169316, 129.2 ± 6.0* ng/mg protein, as measured after 12 h stimulation, $*P < 0.05$). In this study, we found that the viability of the cells treated with 30 μM PD98059, 30 μM SB203580 or 10 μM PD169316 for 13 h was more than 90%, as assessed by trypan blue staining.

3.6. *Effects of PD98059 and SB203580 on the mRNA level for HSP27 in response to TGF-β in MC3T3-E1 cells*

We further examined the effects of PD98059 and SB203580 on the TGF-β-induced increase in the level of mRNA for HSP27 in MC3T3-E1 cells. Both PD98059 and SB203580 significantly suppressed the TGF-β-increased level of mRNA for HSP27 (Fig. 6).

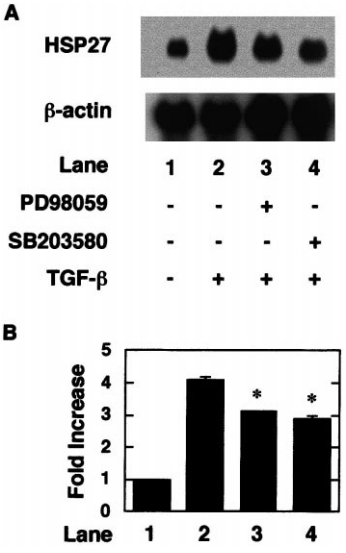
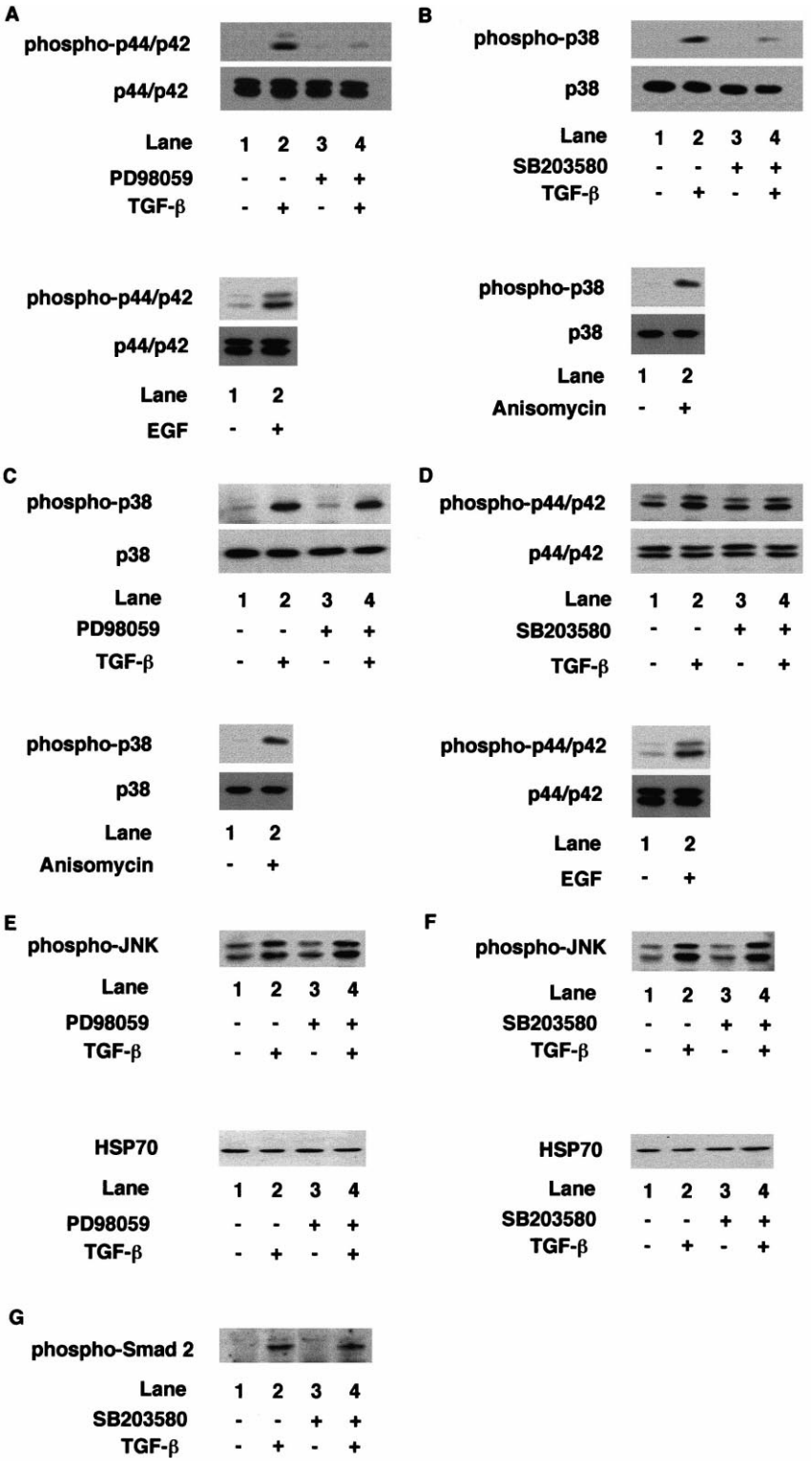


Fig. 6. Effects of PD98059 and SB203580 on the mRNA levels for HSP27 in response to TGF-β in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059, 30 μM SB203580 or vehicle for 60 min, and then stimulated by 3 ng/ml TGF-β or vehicle for 4 h. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample were subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with the cDNA probe for HSP27 or β-actin. Bands of β-actin are shown for reference. The histogram shows quantitative representations of TGF-β-induced HSP27 mRNA levels obtained from laser densitometric analysis after normalization to the levels of β-actin mRNA of three independent experiments. Each value represents the mean ± S.D. of three different sample sets. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of TGF-β alone.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HSP70
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PD98059 - - + +
TGF-β - + - +
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Fig. 7. Effects of PD98059 on the TGF-β-induced phosphorylation of p44/p42 MAP kinase and of SB203580 on the TGF-β-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 (A,C,E) or 30 μM SB203580 (B,D,F) for 60 min, and then stimulated by 3 ng/ml TGF-β or vehicle for 120 min or 12 h (HSP70). Extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies (A,D), against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies (B,C), against phospho-specific JNK antibodies, HSP70 antibodies (E,F), or phospho-specific Smad 2 antibodies (G). Each lower blot shows the positive control for the EGF-induced phosphorylation of p44/p42 MAP kinase or the anisomycin-induced phosphorylation of p38 MAP kinase (A–D).
←

According to densitometric analysis after normalization to the level of β-actin mRNA, PD98059 caused 33% reduction and SB203580 caused 39% reduction in the TGF-β-increased level of HSP27 mRNA.

3.7. *Effects of PD98059 and SB203580 on TGF-β-induced phosphorylation of MAP kinases in MC3T3-E1 cells*

We showed that PD98059 actually inhibited the TGF-β-induced phosphorylation of p44/p42 MAP kinase (Fig. 7A). In addition, SB203580 suppressed the phosphorylation of p38 MAP kinase stimulated by TGF-β (Fig. 7B). However, PD98059 did not affect the phosphorylation of p38 MAP kinase induced by TGF-β (Fig. 7C) and SB203580 had little effect on the TGF-β-stimulated p44/p42 MAP kinase phosphorylation (Fig. 7D). In addition, we examined the effect of PD98059 and SB203580 on the TGF-β-induced phosphorylation of JNK. PD98059 and SB203580 did not affect the TGF-β-induced phosphorylation of JNK (Fig. 7E,F). Furthermore, both PD98059 and SB203580 had little effect on the level of HSP70 (Fig. 7E,F). We showed the phosphorylation of p44/p42 MAP kinase by EGF and the phosphorylation of p38 MAP kinase by anisomycin as loading positive controls.

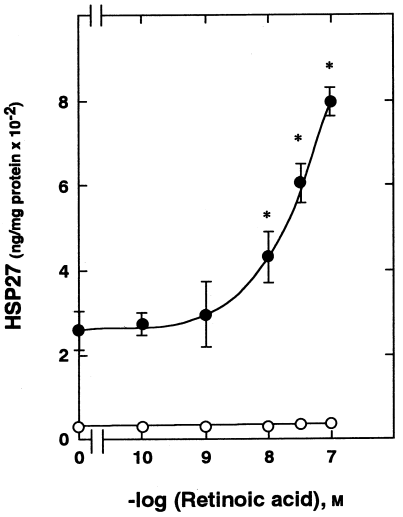

Fig. 8. Effect of retinoic acid on TGF-β-induced HSP27 accumulation in MC3T3-E1 cells. The cultured cells were pretreated with various doses of retinoic acid for 8 h, and then stimulated by 3 ng/ml TGF-β (●) or vehicle (○) for 12 h. Each value represents the mean ± S.D. of three different sample sets. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of TGF-β alone.

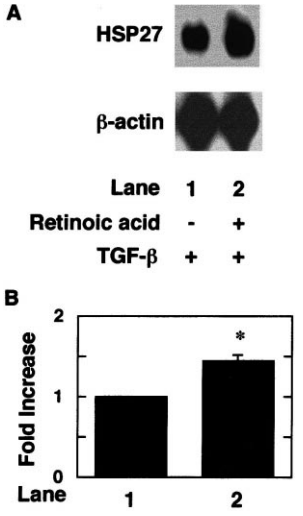

Fig. 9. Effect of retinoic acid on the level of mRNA for HSP27 by TGF-β in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM retinoic acid or vehicle for 8 h, and then stimulated by 3 ng/ml TGF-β for 2 h. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample were subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with the cDNA probe for HSP27 or β-actin. Bands of β-actin are shown for reference. The histogram shows quantitative representations of TGF-β-induced HSP27 mRNA level obtained from laser densitometric analysis after normalization to the levels of β-actin mRNA of three independent experiments. Each value represents the mean ± S.D. of three different sample sets. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of TGF-β alone.

It has been reported that SB203580 can also inhibit kinase activation of TGF-β type I receptor [37]. Thus, we next examined the effect of SB203580 on the phosphorylation of Smad 2 induced by TGF-β. SB203580 (30 μM) failed to affect the phosphorylation of Smad 2 stimulated by TGF-β (Fig. 7G).

3.8. *Effect of retinoic acid on TGF-β-induced HSP27 accumulation in MC3T3-E1 cells*

In order to clarify the interaction between the retinoic acid signaling and the TGF-β signaling system in MC3T3-E1 cells, we examined the effect of retinoic acid on the TGF-β-stimulated HSP27 accumulation. Retinoic acid, which alone had little effect on the basal level of HSP27, significantly enhanced the TGF-β-induced HSP27 accumulation in the range between 0.1 nM and 0.1 μM. The maximal effect of retinoic acid on the HSP27 accumulation by TGF-β was observed at 0.1 μM, a dose that caused about 200% amplification in the effect of TGF-β alone (Fig. 8).

3.9. *Effect of retinoic acid on the mRNA level for HSP27 in response to TGF-β in MC3T3-E1 cells*

We next examined the effect of retinoic acid on the TGF-β-induced increase in the level of mRNA for HSP27 in MC3T3-E1 cells. Retinoic acid significantly amplified the TGF-β-increased level of mRNA for HSP27 as well as the HSP27 accumulation by a specific immunoassay (Fig. 9A). According to densitometric analysis after normalization to the level of β-actin mRNA, retinoic acid caused about 40% amplification in the TGF-β-increased level of HSP27 mRNA (Fig. 9B).

3.10. *Effects of PD98059 and SB203580 on retinoic acid amplification of TGF-β-induced HSP27 accumulation in MC3T3-E1 cells*

In addition, we examined the effects of PD98059 and SB203580 on the enhancement of TGF-β-induced HSP27 accumulation by retinoic acid. PD98059 and SB203580 markedly suppressed the retinoic acid amplification of TGF-β-induced HSP27 accumulation as well as the HSP27 accumulation by TGF-β alone (Fig. 10).

3.11. *Effects of retinoic acid on the TGF-β-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells*

We next investigated whether or not retinoic acid affects the activation of p44/p42 MAP kinase and p38 MAP kinase stimulated by TGF-β. Retinoic acid did neither affect the TGF-β-induced phosphorylation of p44/p42 MAP kinase nor that of p38 MAP

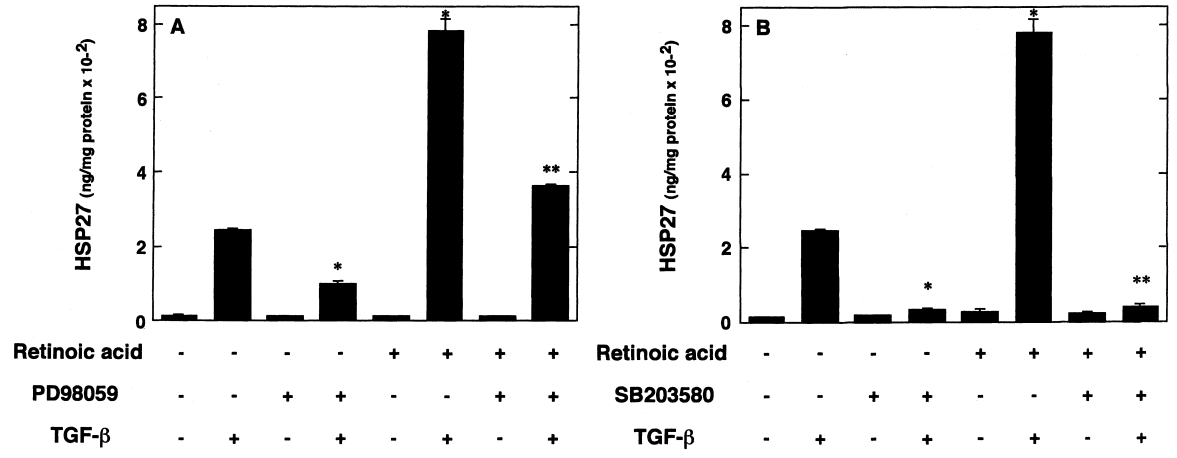

Fig. 10. Effects of PD98059 and SB203580 on the enhancement of TGF-β-induced HSP27 accumulation by retinoic acid in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM retinoic acid or vehicle for 8 h, and then 30 μM PD98059 (A) or 30 μM SB203580 (B) for 60 min, and stimulated by 3 ng/ml TGF-β (●) or vehicle (○) for 12 h. Each value represents the mean ± S.D. of three different sample sets. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of TGF-β alone. $**P < 0.05$, compared to the value of TGF-β with retinoic acid pretreatment.

kinase (Fig. 11). We showed the phosphorylation of p44/p42 MAP kinase by EGF and the phosphorylation of p38 MAP kinase by anisomycin as loading positive controls.

3.12. Effect of TGF-β on the aggregated form of heat-induced HSP27 in MC3T3-E1 cells

It is recognized that HSP27 exists in two forms, an aggregated form and a dissociated form [38]. It has been reported that the dissociation of HSP27 occurs concomitantly with the phosphorylation of HSP27 [38]. Our specific immunoassay of HSP27 detects both an aggregated form and a dissociated form, as previously described [38]. It has been reported that TGF-β phosphorylates HSP27 in MC3T3-E1 cells, analyzed by two-dimensional gel electrophoresis [21]. We investigated the response to TGF-β of the aggregated form of heat-induced HSP27. Extracts of heat-exposed MC3T3-E1 cells contained both forms, an aggregated form and a dissociated form. We found that TGF-β increased the dissociated form while decreasing the aggregated form (data not shown).

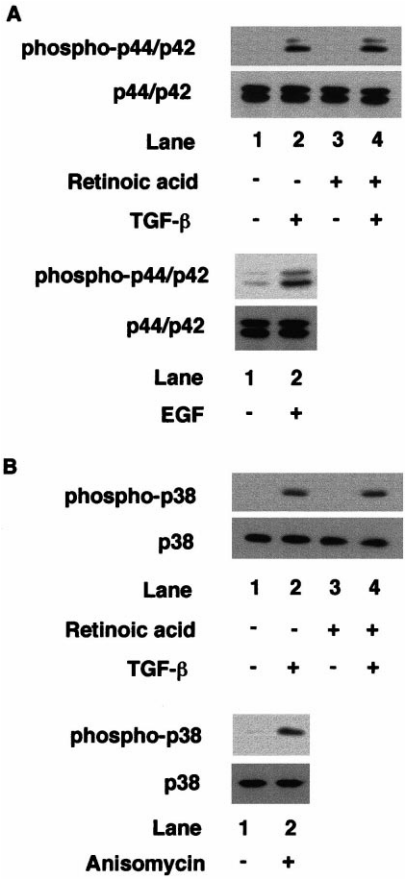

Fig. 11. Effects of retinoic acid on the TGF-β-stimulated phosphorylation of p44/p42 MAP kinase or p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM retinoic acid or vehicle for 8 h, and then stimulated by 3 ng/ml TGF-β or vehicle for 120 min. (A) Extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies. (B) Extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. Each lower blot shows the positive control for the EGF-induced phosphorylation of p44/p42 MAP kinase or the anisomycin-induced phosphorylation of p38 MAP kinase.

4. Discussion

In the present study, we demonstrated that TGF-β stimulated the level of HSP27 in osteoblast-like MC3T3-E1 cells, as detected by Western blotting analysis and specific enzyme immunoassay. In addition, we showed that TGF-β increased the expression level of mRNA for HSP27. Therefore, our findings suggest that TGF-β, as a physiological agonist for osteoblasts, stimulates the induction of HSP27, a low molecular weight HSP, in osteoblast-like MC3T3-E1 cells.

The MAP kinase superfamily is well recognized to play important roles in intracellular signaling of a variety of agonists [13]. In the present study, we showed that TGF-β induced the phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. It is well known that MAP kinase is activated by phosphorylation on both threonine and tyrosine residues by a dual specificity kinase [39]. Thus, it is most likely that TGF-β activates p44/p42 MAP kinase in these cells. To clarify whether p44/p42 MAP kinase activation is involved in the induction of HSP27 stimulated by TGF-β in MC3T3-E1 cells, the effect of PD98059 on HSP27 accumulation was next investigated. PD98059 significantly reduced the HSP27 accumulation by TGF-β. We found that the TGF-β-induced phosphorylation of p44/p42 MAP kinase was truly inhibited by PD98059. In addition, the TGF-β-increased level of mRNA for HSP27 was markedly reduced by PD98059. Therefore, our findings suggest that p44/p42 MAP kinase activation is involved in

the TGF-β-stimulated induction of HSP27 in osteoblast-like MC3T3-E1 cells.

p38 MAP kinase is another member of the MAP kinase superfamily [13]. As for p38 MAP kinase in osteoblasts, SK&F 86002, a cytokine-suppressant anti-inflammatory drug, has been shown to inhibit cytokine-stimulated IL-6 synthesis through p38 MAP kinase in cultured neonatal mouse calvaria and Saos-2 osteoblastic cells [40]. It has been reported that the proliferation of osteoblasts in response to EGF or hypoxia is associated with the activation of not p38 MAP kinase but p44/p42 MAP kinase [41]. We previously reported that IL-1α-induced IL-6 synthesis is mediated via activation of both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells [42]. Thus, we next investigated whether or not TGF-β stimulates the activation of p38 MAP kinase in MC3T3-E1 cells. The phosphorylation of p38 MAP kinase was significantly stimulated by TGF-β. Our finding suggests that TGF-β activates p38 MAP kinase in MC3T3-E1 cells. In addition, SB203580 and PD169316 markedly suppressed the HSP27 accumulation by TGF-β. We found that SB203580 truly inhibited the TGF-β-induced phosphorylation of p38 MAP kinase. Therefore, it is probable that p38 MAP kinase is involved in the TGF-β-stimulated HSP27 induction in MC3T3-E1 cells. Additionally, SB203580 also reduced the TGF-β-increased level of mRNA for HSP27. It has been reported that SB203580 can also inhibit the kinase activity of TGF-β type I receptor [37]. It is generally recognized that the activated TGF-β type I receptor phosphorylates Smad 2 and Smad 3, which mediate the effect of TGF-β [7,8]. Thus, we examined the effect of SB203580 on the phosphorylation of Smad 2 in MC3T3-E1 cells and showed that SB203580 failed to affect the phosphorylation of Smad 2 induced by TGF-β. Based on our findings, it is most likely that p38 MAP kinase activation in addition to p44/p42 MAP kinase activation is necessary for the TGF-β-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. However, each inhibitory effect of PD98059 and SB203580 on the TGF-β-increased HSP27 mRNA was partial. Thus, these findings make us speculate that the combination of p38 MAP kinase and p44/p42 MAP kinase accounts for the entire induction of HSP27 in response to TGF-β in MC3T3-E1 cells. In addition, it is likely that other signaling pathways are involved in TGF-β-stimulated HSP27 induction. In our previous studies [43,44], we have shown that not p44/p42 MAP kinase but p38 MAP kinase is involved in endothelin-1- and sphingosine 1-phosphate-stimulated HSP27 induction in these cells. These differences in the signaling of HSP27 induction may be due to both agonists. Further investigations are necessary to clarify the details.

It has been reported that p44/p42 MAP kinase activated by EGF or hepatocyte growth factor inhibits the signaling of BMP-2, a member of the TGF-β superfamily, via the phosphorylation of Smad 1 [45]. On the other hand, we here showed that the TGF-β-stimulated p44/p42 MAP kinase activation acts as a positive regulator in the TGF-β itself-induced HSP27 in MC3T3-E1 cells. Further investigations would be required to clarify the relationship between the p44/p42 MAP kinase signaling and the Smad signaling in the TGF-β-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells since the role of Smad signaling in the HSP27 induction by TGF-β is unclear.

The evidence of an implication between p38 MAP kinase and HSP27 is accumulating in several cells, showing that the activation of p38 MAP kinase leads to the phosphorylation of HSP27 [18]. HSP27 exists in two forms, an aggregated form and a dissociated form. It is recognized that the dissociation of HSP27 occurs concomitantly with the phosphorylation of HSP27, as described previously [38]. We showed that HSP27 levels were low in unstimulated MC3T3-E1 cells. Thus, we examined the possible existence of two forms of heat-induced HSP27 in these cells. Extracts of heat-exposed cells contained both forms, an aggregated form and a dissociated form. We found that TGF-β increased the dissociated form while decreasing the aggregated form. It has been reported that TGF-β phosphorylates HSP27 in MC3T3-E1 cells [21]. Taking these findings into account, it is most likely that TGF-β not only phosphorylates HSP27 but also stimulates its induction in osteoblast-like MC3T3-E1 cells.

It is well recognized that high molecular weight HSPs such as HSP70 act as molecular chaperones [18]. It is speculated that low molecular weight HSPs such as HSP27 may also act as chaperones like high molecular weight HSPs [18]. It has been

shown that heat stress induces both HSP70 and HSP27 in osteoblasts [19]. In the present study, TGF-β did not affect the level of HSP70 in MC3T3-E1 cells while increasing the level of HSP27. The HSP70 family is composed of HSC70 and inducible HSP70. For the detection of 70 kDa HSP, we used antibodies for inducible HSP70, which are non-cross-reactive with HSC70. It is generally recognized that inducible HSP70 usually is not expressed in cells under normal conditions. It has been shown that HSC70 is induced by TGF-β in chicken embryonic fibroblasts [46]. On the contrary, we found that inducible HSP70 was expressed in MC3T3-E1 cells under normal conditions and the level of HSP70 was not affected by TGF-β. These findings lead us to speculate that HSP27 induced by TGF-β may play an important role in osteoblast functions following the stimulation by TGF-β.

TGF-β reportedly stimulates proliferation of osteoblast-like MC3T3-E1 cells [47]. In addition, it has been shown that the expression of HSP27 mRNA increases with the downregulation of proliferation in osteoblasts [19]. These findings made us speculate that HSP27 expression may be necessary for the inhibition of osteoblast proliferation. In addition, it has been reported that TGF-β increases the synthesis of bone matrix via the activation of p44/p42 MAP kinase and p38 MAP kinase in osteoblasts [5,48]. We showed that TGF-β stimulated HSP27 induction via the activation of both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. Based on these findings, it is possible that the induction of HSP27 by TGF-β may play an important role in inducing osteoblastic bone formation.

In addition, it has been reported that overexpression of HSP27 at biopsy in patients with osteosarcoma is associated with a poor prognosis [49]. Investigations of HSPs, especially HSP27, in osteoblasts suggest that HSP27 may contribute to physiological and pathological processes such as osteosarcoma. Further investigations concerned with HSP27 in osteoblasts are necessary to clarify the exact role of HSP27 in bone metabolism.

It has been reported previously that the ligand-dependent interaction between Smad 3 and retinoid X receptor/vitamin D receptor heterocomplex is observed and the cooperative actions of TGF-β and vitamin D signaling pathways are mediated by Smad 3 [27]. Thus, it is speculated that TGF-β and various steroid hormones cooperatively regulate cell function in some cell types. In the present study, retinoic acid, which alone did not affect the level of HSP27, enhanced the TGF-β-induced HSP27 accumulation in osteoblast-like MC3T3-E1 cells. In addition, we showed that retinoic acid, which by itself had little effect on the mRNA level for HSP27, amplified the TGF-β-induced increase in the mRNA for HSP27. Therefore, our findings suggest that retinoic acid acts as an enhancer in the TGF-β-induced HSP27 in osteoblast-like MC3T3-E1 cells. We next showed that PD98059 and SB203580 also inhibited the retinoic acid enhancement of TGF-β-stimulated HSP27 accumulation. Furthermore, retinoic acid failed to affect the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase stimulated by TGF-β. Therefore, it is probable that the effect of retinoic acid on the HSP27 induction stimulated by TGF-β is exerted at a point downstream from p44/p42 MAP kinase and p38 MAP kinase.

TGF-β is known to be an osteoinductive agent while retinoic acid is recognized to act as a bone resorptive agent. It has been reported that overexpression of TGF-β2 in osteoblasts causes an osteoporotic-like phenotype [50]. An increased osteoblastic expression of TGF-β causes both an increase in bone remodeling and an imbalance in osteoblastic and osteoclastic activity leading to progressive bone loss, a phenotype similar to high-turnover osteoporosis. In the present study, we showed that TGF-β-stimulated HSP27 induction was amplified by the presence of retinoic acid, which alone did not affect HSP27 induction. These findings made us speculate that the overexpression of the TGF-β-stimulated HSP27 by retinoic acid in osteoblasts may upregulate bone resorption. It is generally recognized that the chaperoning function for most of the HSPs such as HSP27 is their main function. In addition, intensive deposition of extracellular matrix is observed during bone remodeling. It has been reported that TGF-β causes an increase in the steady state level of HSP47 mRNA in osteoblast-like MC3T3-E1 cells [22]. HSP47 is a procollagen/collagen-specific molecular chaperone protein. These findings make us speculate that TGF-β-induced HSP27 assists the folding of newly synthesized bone matrix proteins by TGF-β. In addition, it is possible that retinoic acid might

strengthen the TGF-β effect via potentiation of HSP27 induction.

In conclusion, these results strongly suggest that TGF-β stimulates HSP27 induction through p44/p42 MAP kinase activation and p38 MAP kinase activation in osteoblasts, and that retinoic acid up-regulates the TGF-β-stimulated HSP27 induction as an amplifier at a point downstream from p44/p42 MAP kinase and p38 MAP kinase.

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